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NEW APPLICATION TRANSMITTAL - UTILITY

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Transmitted herewith for filing is a **utility** patent application:

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Title: PHARMACEUTICAL COMPOSITIONS AND METHODS TO VACCINATE AGAINST DISSEMINATED CANDIDIASIS

I.   PAPERS ENCLOSED HEREWITH FOR FILING UNDER 37 CFR § 1.53(b):

<u>23</u>   Page(s) of Written Description

<u>2</u>   Page(s) Claims

<u>1</u>   Page(s) Abstract

<u>14</u>   Sheets of Drawings   ☒ Informal   ☐ Formal

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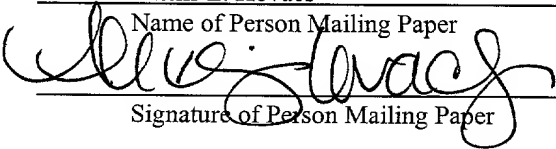☐   Preliminary Amendment

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Respectfully submitted,

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Dated: <u>November 18, 2000</u>   By: _____
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PHARMACEUTICAL COMPOSITIONS AND METHODS

TO VACCINATE AGAINST DISSEMINATED CANDIDIASIS

RELATED INFORMATION

5   This application claims priority from Provisional Application Serial No.: 60/166,663

filed November 19, 1999. This invention was made with Government support under Public

Health Service grants PO-1AI-37194, RO1AI-19990, and MO1 RR0425. The Government has

certain rights in this invention.

FIELD OF INVENTION

10   This invention relates to *Candida albicans* surface adhesin proteins, to antibodies

resulting from an immune response to vaccination, and to methods for the prevention and/or

treatment of candidiasis.

BACKGROUND OF INVENTION

15   There has been a dramatic increase in the incidence of nosocomial infections caused by

Candida species in recent years. The incidence of hematogenously disseminated candidal infections

increased 11-fold from 1980 to 1989. This increasing incidence has continued into the 1990s.

Infections by *Candida* species are now the fourth most common cause of nosocomial septicemia, are

equal to that of *Escherichia coli*, and surpass the incidence caused by *Klebsiella* species.

20   Furthermore, *Candida* species are the most common cause of deep-seated fungal infections in

patients who have extensive burns. Up to 11% of individuals undergoing bone marrow

transplantation and 13% of those having an orthotopic liver transplant will develop an invasive

candidal infection.

Candida albicans, the major pathogen in this genus, can switch between two morphologies: the blastospore (budding yeast) and filamentous (hyphae and pseudohyphae) phases. *Candida* mutants that are defective in genes regulating filamentation are reported to have reduced virulence in animal models. This reduced virulence suggests that the ability to change from a blastospore to a

5   filament is a key virulence factor of *C. albicans*. To date, no essential effectors of these filamentation pathways have been identified in *C. albicans*. See Caesar-TonThat, T.C. and J.E. Cutler, "A monoclonal antibody to *Candida albicans* enhances mouse neutrophil candidacidal activity," Infect. Immun. 65:5354-5357, 1997.

The identification of effectors in the regulatory pathways of the organism that contribute to

10 virulence offers the opportunity for therapeutic intervention with methods or compositions that are superior to existing antifungal agents. The identification of cell surface proteins that effect a regulatory pathway involved in virulence is particularly promising because characterization of the protein enable immunotherapeutic techniques that are superior to existing antifungal agents when fighting a candidal infection.

15 The virulence of *Candida albicans* is regulated by several putative virulence factors of which adherence to host constituents and the ability to transform from yeast-to-hyphae are among the most critical in determining pathogenicity. While potent antifungal agents exist that are microbicidal for *Candida*, the attributable mortality of candidemia is approximately 38%, even with treatment with potent anti-fungal agents such as amphotericin B. Also, existing agents

20 such as amphotericin B tend to exhibit undesirable toxicity. Although additional antifungals may be developed that are less toxic than amphotericin B, it is unlikely that agents will be developed that are more potent. Therefore, either passive or active immunotherapy to treat or prevent disseminated candidiasis is a promising alternative to standard antifungal therapy.

SUMMARY OF INVENTION

The present invention utilizes the gene product of *C. albicans* agglutinin like sequence ALS1 as a vaccine to treat, prevent, or alleviate disseminated candidiasis. The invention takes advantage of the role of the ALS1 gene product in the adherence of the *C. albicans* to endothelial and epithelial cells and the susceptibility of the ALS1-expressed surface protein for use as a vaccine to retard the pathogenesis of the organism.

Pursuant to this invention, the ALS1 gene encodes a surface adhesin that is selected as the target of an immunotherapeutic strategy against *Candida Albicans*. A demonstration that the expression product of the ALS1 gene, the ALS1p protein, has structural characteristics typical of surface proteins and is, in fact, expressed on the cell surface of *C. albicans* is a critical criterion for proteins that act as adhesins to host tissues. In this case, ALS1p has a signal peptide at the N-terminus, a glycosylphosphatidylinosine (GPI) anchorage sequence in the C-terminus, and a central region comprising repeats rich in threonine and serine. Also, the ALS1 protein has many N-, and O-glycosylation sites, typical of proteins that are expressed on the cell surface. Indirect immunofluorescence using a monoclonal antibody directed against the N-terminus of Als1p revealed that Als1p is expressed during the log phase of blastospores. This expression of Als1p is increased during hyphal formation and is localized to the junction where the hyphal element extends from the blastospores as indicated by the diffused surface staining. Furthermore, this monoclonal antibody blocked the enhanced adherence of *C. albicans* overexpression mutant to endothelial cells, thereby establishing the principle for immunotherapy applications using Als1p.

Additional evidence that Als1p is a surface adhesin protein is based on data showing that antibodies that bind to the surface of *C. albicans* also bind to the surface of *S. cerevisiae* transformed

with ALS1, but not with empty plasmid. The ALS1 protein also shares significant homology with the alpha-agglutinin of *S. cerevisiae,* which is expressed on the cell surface and mediates the binding of mating type alpha cells to mating type a cells. Moreover, expression of the ALS1 gene in *S. cerevisiae* increases the adherence of this organism to endothelial cells by approximately 100-fold.

5   Because the ALS1 gene appears to encode a functional adhesin in S cerevisiae, it is certain that it also encodes a functional adhesin in *C. albicans.* The *ALS1* gene was originally isolated by Hoyer et al. without a known function. Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi. 1995. *Candida albicans ALSI:* domains related to a *Saccharonzyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. 15:39-54. (*See also* US Patents 5,668,263 and 5,817,466.)

10   Thus, according to one aspect of the invention we provide an ALS1 surface adhesin protein, designated Als1p, or a fragment conjugate or analogue thereof, having useful properties when formulated in a pharmaceutical composition and administered as a vaccine. Als1p or functional analogues conjugates or derivatives thereof, is preferably obtained from *Candida albicans.* However, similar adhesin molecules or analogues or derivatives thereof may be of

15   candidal origin and may be obtainable, for example, from strains belonging to the genera *Candida,* for example *Candida parapsilosis, Candida krusei,* and *Candida tropicalis.* A surface adhesin protein according to the invention may be obtained in purified form, and thus, according to a preferred embodiment of the invention a substantially pure ALS1 *Candida albicans* surface adhesin protein, or functional analogue conjugates or derivative thereof, is formulated as a

20   vaccine to cause an immune response in a patent to block adhesion of the organism to the endothelial cells.

An analogue or derivative of the surface adhesion protein according to the invention may be identified and further characterized by the criteria described herein for the ALS1 gene and

gene product. For example, a null mutant of the analogue or derivative would share markedly reduced adhesion to endothelial cells compared to controls. Similarly, over-expression of the analogue or derivative in an appropriate model would show an increased adherence to endothelial cells compared to controls and would be confirmed as a cell surface adhesin in accord with the criteria described above. Also, antisera to the analogue or derivative would cross-react with anti-ALS1 antibodies and would also exhibit increased survival times when administered in a mouse model of disseminated *candidiasis* as disclosed herein.

The present invention also provides an immunotherapeutic strategy against *Candida* infection at the level of binding to the vascular endothelial cells and through a downstream effector of the filamentation regulatory pathway. An immunotherapeutic strategy is uniquely advantageous in this context because: (i) the morbidity and mortality associated with hematogenously disseminated candidiasis remains unacceptably high, even with currently available antifungal therapy; (ii) a rising incidence of antifungal resistance is associated with the increasing use of antifungal agents, iii) the population of patients at risk for serious *Candida* infections is well-defined and very large, and includes post-operative patients, transplant patients, cancer patients and low birth weight infants; and iv) a high percentage of the patients who develop serious *Candida* infections are not neutropenic, and thus may respond to a vaccine. For these reasons, *Candida* is the most attractive fungal target for either passive or active immunotherapy.

Having determined the immunotherapeutic potential of Als1p according to this invention, this protein and conjugates analogues, or derivative molecules thereof may be used in treatment and/or prevention of candidal infections. Standard immunological techniques may be employed with the adhesion protein molecule, and its analogues, conjugates, or derivatives, to use the

molecule as an immunogen in a pharmaceutically acceptable composition administered as a

vaccine. For the purposes of this invention, "pharmaceutical" or "pharmaceutically acceptable"

compositions are formulated by known techniques to be non-toxic and, when desired, used with

carriers or additives that are approved for administration to humans in, for example, intravenous,

5   intramuscular, intraperitoneal or sub-cutaneous injection. Such compositions may include

buffers, salts or other solvents known to these skilled in the art to preserve the activity of the

vaccine in solution.

With respect to the molecule used as the immunogen pursuant to the present invention,

those of skill in the art will recognize that the Als1p molecule may be truncated or fragmented

10   without losing the essential qualities as a vaccine. For example, Als1p may be truncated to yield

an N-terminal fragment by truncation from the C-terminal end with preservation of the

functional properties described above. Likewise, C-terminal fragments may be created by

truncation from the N-terminal end with preservation of the functional properties described

above. Other modifications in accord with the foregoing rationale may be made pursuant to this

15   invention to create other Als1p analogs or derivatives, to achieve the benefits described herein

with the native protein.

The goal of the immunotherapy provided by this invention to interfere with regulation of

filamentation, to block adherence of the organism to host constituents, and to enhance clearance

of the organism by immunoeffector cells. Since endothelial cells cover the majority of the

20   vasculature, strategies to block the adherence of the organism to endothelial cells using

antibodies are a preferred embodiment of the present invention and such adherence blocking

strategies include active or passive immunotherapy directed against the candidal adhesin(s)

disclosed herein. Thus, for example, any suitable host may be injected with protein and the

serum collected to yield the desired anti-adhesin antibody after appropriate purification and/or concentration. Prior to injection, the adhesin protein may be formulated in a suitable vehicle, preferably a known immunostimulant such as a polysaccharide. Thus, according to a further aspect of the invention we provide a pharmaceutical composition comprising a candidal adhesin

5   protein together with one or more pharmaceutically acceptable excipients in a formulation for use as a vaccine.

The method of the invention is ameliorating and/or preventing candidal infection by blocking the adherence of *C. albicans* to the endothelial cells of a host constituent. Thus, according to one aspect of the invention, a pharmaceutical composition comprising an ALS1

10   adhesin protein derivative, analogue, or conjugate is formulated as a vaccine in a pharmaceutical composition containing a biocompatible carrier for injection or infusion and is administered to a patient. Also, direct administration of antiserum raised against ALS1 protein may be used to block the adherence of *C. albicans* to a mammalian host constituent. Antiserum against adhesin protein can be obtained by known techniques, Kohler and Milstein, Nature 256: 495-499 (1975),

15   and may be humanized to reduce antigenicity, see USP 5,693,762, or produced in transgenic mice leaving an unrearranged human immunoglobulin gene, see USP 5,877,397.

A still further use of the invention, for example, is using the ALS1 adhesin protein to develop vaccine strategies for the prevention and/or amelioration of candidal infections. Thus, according to one aspect of the invention, for example, standard immunology techniques may be

20   employed to construct a multi-component vaccine strategy that may enhance and/or elicit immune response from a host constituent to bock adherence of *C. albicans*.

A still further use of the invention, for example, is developing DNA vaccine strategies. Thus, according to one aspect of the invention, for example, the ALS1 polynucleotide encoding

Als1p on a fragment thereof is administered according to a protocol designed to yield an immune response to the gene product. See e.g., Felgner USP 5,703,055.

A still further use of the invention, for example, is developing combination vaccine strategies. Thus, according to one aspect of the invention, for example, anti-ALS antibodies may

5   be used with antibodies in treating and/or preventing candidal infections. See USP 5,578,309.

DESCRIPTION OF THE FIGURES

Figure 1A, 1B show the mediation of Als1p adherence of $C.$ $albicans$ to human umbilical vein endothelial cells. Values represent the mean $\pm$ SD of at least three independent

10   experiments, each performed in triplicate. (A) Endothelial cell adherence of $ALS1/als2$, $als1/als1$ and $ALS1$-complemented mutants and wild-type CAI12 (30) (B) Endothelial cell adherence of P_{ADH1}-ALS1 mutant that overexpresses $ALS1$, compared to wild type $C.$ $albicans$. Statistical treatment was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction. $*P<0.001$ for all comparisons.

15   Figure 2A-D shows the cell surface localization of Als1p on filaments of $C.$ $albicans$ by indirect immunofluorescence. Filamentation of $C.$ $albicans$ was induced by incubating yeast cells in RPMI 1640 medium with glutamine for 1.5 hours at 37°C. Als1p was detected by incubating organisms first with anti-Als1p mouse mAb followed by FITC-labeled goat anti-mouse IgG. $C.$ $albicans$ cell surface was also stained with anti-$C.$ $albicans$ polyclonal Ab

20   conjugated with Alexa 594 (Molecular Probes, Eugene, OR). Areas with yellow staining represent Als1p localization. (A) $C.$ $albicans$ wild-type. (B) $als1/als1$ mutant strain. (C) $als1/als1$ complemented with wild type $ALS1$ (D) P_{ADH1}-ALS1 overexpression mutant.

Figure 3A, 3B show the mediation of Als1p on *C. albicans* filamentation on solid

medium. *C. albicans* blastospores were spotted on Lee's agar plates and incubated at 37°C for 4

days (A) or 3 days (B).

Figure 4A, 4B show the control of *ALS1* expression and the mediation of *C. albicans*

5   filamentation by the *EFG1* filamentation regulatory pathway. (A) Northern blot analysis

showing expression of *ALS1* in (i) mutants deficient in different filamentation regulatory

pathways. (ii) *efg1/efg1* mutant complemented with either *EFG1* or P_{ADH1}-*ALS1*. Total RNA

was extracted from cells grown in RPM1 1640 + glutamine medium at 37°C for 90 minutes to

induce filamentation. Blots were probed with *ALS1* and *TEF1*. (B) Photomicrographs of the

10   *efg1/efg1* mutant and *efg1/efg1* mutant complemented with P_{ADH1}-*ALS1* grown on Lee's agar

plates at 37°C. for 4 days.

Figure 5A, 5B show the reduction of virulence in the mouse model of hematogenously

disseminated candidiasis by (A) Male Balb/C mice ($n = 30$ for each yeast strain) were injected

with stationary phase blastospores (10^6 per mouse in 0.5 ml of PBS). Curves are the compiled

15   results of three replicate experiments ($n = 30$ mice for each strain). The doubling times of all

strains, grown in YPD at 30°C, ranged between 1.29 to 1.52 hours and were not statistically

different from each other. Southern blot analysis of total chromosomal DNA was used to match

the identity of the genotype of *C. albicans* strains retrieved from infected organs with those of *C.*

albicans strains used to infect the mice. Statistical analysis was obtained by Wilcoxon rank sum

20   test and corrected for multiple comparisons with the Bonferroni correction. *P <0.002 for the

als1/als1 mutant versus each of the other strains. (B) Histological micrographs of kidneys

infected with *C. albicans* wild-type, homozygous *als1 null* mutant, or heterozygous *ALS1*

complemented mutant. Kidney samples were retrieved 28 hours (a) or 40 (b) hours post

infection, fixed in paraformaldehyde and sections were stained with silver (magnification,

X400). Arrows denote *C. albicans* cells.

Figure 6 shows the prophylactic effect of anti-ALS antibody against disseminated

candidiasis as a function of surviving animals over a 30-day period for animals infused with anti-

5   Als1p polyserum.

Figure 7 is the polypeptide sequence of Als1p.

DETAILED DESCRIPTION OF THE INVENTION

The nature of the pathogenesis of *C. albicans* by adherence to endothelial cells is

10   discussed in USP 5,578,309 which is specifically incorporated herein by reference in its entirety.

For a description of the ALS1 gene and characteristics thereof, including the characterization of

the gene product as an adhesin, see Fu, Y., S. G. Filler, B. J. Spellberg, W. Fonzi, A. S. Ibrahim,

T. Kanbe, M. A. Ghannoum, and J. E. J. Edwards . 1998. Cloning and characterization of CAD

I/AAF1, a gene from *Candida albicans* that induces adherence to endothelial cells after

15   expression *in Saccharonzyces cerevisiae*. Infect. Immun. 66:2078-2084; Fu, Y., G. Rieg, W. A.

Forizi, P. H. Belanger, J. E. J. Edwards, and S. G. Filler. 1998. Expression of the *Candida*

albicans gene ALS1 in Saccharomyces cerevisiae induces adherence to endothelial and epithelial

cells. Infect. Immun. 66:1783-1786; Hoyer, L.L. 1997. The ALS gene family of *Candida*

albicans. International Society for Human and Animal Mycology Salsimorge, Italy:(Abstract);

20   Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi. 1995. *Candida albicans ALS1:*

domains related to a *Saccharonzyces cerevisiae* sexual agglutinin separated by a repeating motif.

Mol. Microbiol. 15:39-54.

The following Examples illustrate the immunotherapeutic utility of the ALS1 adhesin as

the basis for preventive measures or treatment of disseminated candidiasis. Example 1 describes

the preparation of an ALS1 null mutant and a strain of *C. albicans* characterized by over-

expression of ALS1 to confirm the mediation of adherence to endothelial cells. Example 2

5   describes the localization of Als1p and the implication of the efg filamentation regulatory

pathway. Example 3 describes the purification of ALS1 adhesin protein. Example 4 describes

the preparation of rabbit polyclonal antibodies raised against the ALS1 surface adhesin protein to

be used to demonstrate the blocking of the surface adhesin protein. Example 5, describes the

blocking of adherence <u>in</u> <u>vivo</u>, using polyclonal antibodies raised against the ALS1 surface

10   adhesion protein as described herein according to the invention to protect against disseminated

candidiasis in a mouse model.

EXAMPLE 1 – Als1 Mediates Adherence of *C. albicans* to Endothelial Cells

The URA blaster technique was used to construct a null mutant of C *albicans* that lacks

expression of the Als1p. The *als1/als1* mutant was constructed in *C. albicans* strain CAI4 using

15   a modification of the Ura-blaster methodology [W. A. Fonzi and M. Y. Irwin, *Genetics* 134, 717

(1993)] as follows: Two separate *als1-hisG-IRA3-hisG-als1* constructs were utilized to disrupt

the two different alleles of the gene. A 4.9 kb *ALS1* coding sequence was generated with high

fidelity PCR (Boehringer Mannheim, Indianapolis, IN) using the primers: 5'-

20   CCGCTCGAGATGCTTCAACAATTTACATTGTTA-3' and 5'-

CCGCTCGAGTCACTAAATGAACAAGGACAATA3'. Next, the PCR fragment was cloned

into pGEM-T vector (Promega, Madison, WI), thus obtaining pGEM-T-*ALS1*. The *hisG-URA3-*

hisG construct was released from pMG-7 by digestion with *KpnI* and *Hind3* and used to replace

the portion of *ALS1* released by *Kpn1* and *Hind3* digestion of pGEM-T-*ALS1*. The final *als1-hisG-URA3-hisG-als1* construct was released from the plasmid by digestion with *Xho1* and used to disrupt the first allele of *ALS1* by transformation of strain CAI-4.

A second *als1-hisG-URA3-hisG-als1* construct was generated in two steps. First, a *Bgl2-Hind3 hisG-URA3-hisG* fragment of pMB7 was cloned into the *BamH1-Hind3* sites of pUC19,

5 thereby generating pYC2. PYC2 was then digested with *Hind3*, partially filled in with dATP and dGTP using T4 DNA polymerase, and then digested with *Sma1* to produce a new *hisG-URA3-hisG* fragment. Second, to generate *ALS1* complementary flanking regions, pGEM-T-*ALS1* was digested with *Xba1* and then partially filled in with dCTP and dTTP. This fragment

10 was digested with *Hpa1* to delete the central portion of *ALS1* and then ligated to the *hisG-URA3-hisG* fragment generating pYC3. This plasmid was then digested by *Xho1* to release a construct that was used to disrupt the second allele of the *ALS1*. Growth curves were done throughout the experiment to ensure that the generated mutations had no effect on growth rates. All integrations were confirmed by Southern blot analysis using a 0.9kb *ALS1* specific probe generated by

15 digestion of pYF5 with *Xba1* and *HindIII*.

The null mutant was compared to *C. albicans* CAI-12 (a URA + revertant strain) for its ability to adhere in vitro to human umbilical vein endothelial cells. For adherence studies, yeast cells from YPD (2% glucose, 2% peptone, and 1% yeast extract) overnight culture, were grown in RPMI with glutamine at 25°C for 1 hour to induce Als1p expression. 3×10^2 organisms in

20 Hanks balanced salt solution (HBSS) (Irvine Scientific, Irvine, CA) were added to each well of endothelial cells, after which the plate was incubated at 37°C for 30 minutes. The inoculum size was confirmed by quantitative culturing in YPD agar. At the end of incubation period, the nonadherent organisms were aspirated and the endothelial cell monolayers were rinsed twice

with HBSS in a standardized manner. The wells were over laid with YPD agar and the number

of adherent organisms were determined by colony counting. Statistical treatment was obtained

by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni

correction. $P<0.001$.

5   Referring to Figure 1, a comparison of the ALS1/ALS1 and als1/als1 strain showed that

the ALS1 null mutant was 35% less adherent to endothelial cells than *C. albicans* CAI-12. To

reduce background adherence, the adherence of the wild-type strain grown under non-ALS1

expressing conditions was compared with a mutant autonomously expressing Als1p. This

mutant was constructed by integrating a third copy of ALS1 under the control of the constitutive

10   ADH1 promoter into the wild-type *C.* albicans. To achieve constitutive expression of the ALS1

in *C. albicans,* a blunt-ended PCR generated *URA3* gene is ligated into a blunt-edged Bg12 site

of pOCUS-2 vector (Novagen, Madison, WI), yielding pOU-2. A 2.4 kb *Not1-Stul* fragment,

which contained *C. albicans* alcohol dehydrogenase gene (ADH1) promoter and terminator

(isolated from pLH-ADHpt, and kindly provided by A. Brown, Aberdeen, UK), was cloned into

15   pOU-2 after digestion with *Not1* and *Stul*. The new plasmid, named pOAU-3 had only one *Bg12*

site between the *ADH1* promoter and terminator. *ALS1* coding sequence flanked by *BamH1*

restriction enzyme sites was generated by high fidelity PCR using pYF-5 as a template and the

following primers: 5'-CGGGATCCAGATGCTTCA-ACAATTTACATTG-3' and 5'-

CGGGATCCTCACTAAATGAACAAGGACAATA-3'. This PCR fragment was digested with

20   *BamH1* and then cloned into the compatible *Bg12* site of pOAU-3 to generate pAU-1. Finally,

pAU-1 was linearized by *Xbal* prior to transforming *C. albicans* CAI-4. The site-directed

integration was confirmed by Southern Blot analysis. Referring to Figure 1B, overexpressing

ALS1 in this P_{ADH1}-*ALS1* strain resulted in a 76% increase in adherence to endothelial cells,

compared to the wild-type *C. albicans*. In comparing endothelial cell adherence of the wild-type

to that of the overexpressing mutant, yeast cells were grown overnight in YPD at 25°C (non-

inducing condition of Als1p). Als1p expression was not induced to reduce the background

adherence of the wile-type, thus magnifying the role of Als1p in adherence through P_{ADH1}-*ALS1*

5   hybrid gene. The adherence assay was carried out as described above. Statistical treatment was

obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni

correction. *P<0.001*.

A monoclonal anti-Als1p murine IgG antibody was raised against a purified and

truncated N-terminus of Als1p (amino acid #17 to #432) expressed using Clontech YEXpress

10   (™) Yeast Expression System (Palo Alto, CA). The adherence blocking capability of these

monoclonal anti-Als1p antibodies was assessed by incubating *C. albicans* cells with either anti-

Als1 antibodies or mouse IgG (Sigma, St. Louis, MO) at a 1:50 dilution. After which the yeast

cells were used in the adherence assay as described above. Statistical treatment was obtained by

Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction.

15   *P<0.001*. The results revealed that the adherence of the P_{ADH1}-ALS1 strain was reduced from

26.8%± 3.5% to 14.7%± 5.3%. Thus, the effects of ALS1 deletion and overexpression

demonstrate that Als1p mediates adherence of *C. albicans* to endothelial cells.

EXAMPLE 2 – Localization of Als1p

20   For Als1p to function as an adhesin, it must be located on the cell surface. The cell

surface localization of Als1p was verified using indirect immunofluorescence with the anti-

Als1p monoclonal antibody. Diffuse staining was detected on the surface of blastospores during

exponential growth. This staining was undetectable on blastospores in the stationary phase.

Referring to Figure 2A, when blastospores were induced to produce filaments, intense staining

was observed that localized exclusively to the base of the emerging filament. No

immunofluorescence was observed with the *als1/als1* mutant, confirming the specificity of this

antibody for Als1p. See Figure 2B. These results establish that Als1p is a cell surface protein.

5   The specific localization of Als1p to the blastospore-filament junction implicates Als1p

in the filamentation process. To determine the mechanism, the filamentation phenotype of the *C.*

albicans ALS1 mutants was analyzed. Referring to Figure 3A, the *als1/als1* mutant failed to

form filaments after a 4 day incubation on Lee's solid medium, while both the *ALS1/ALS1* AND

ALS1/als1 strains as well as the *ALS1*-complemented mutant produced abundant filaments at this

10   time point. The *als1/als1* mutant was capable of forming filaments after longer periods of

incubation. Furthermore, overexpressing *ALS1* augmented filamentation: the P_{ADH1}-ALS1 strain

formed profuse filaments after a 3 day incubation, whereas the wild-type strain produced scant

filaments at this time point. See Figure 3B. To further confirm the role of Als1p in

filamentation, a negative control was provided using mutant similar to the *ALS1* overexpression

15   mutant, except the coding sequence of the *ALS1* was inserted in the opposite orientation. The

filamentation phenotype of the resulting strain was shown to be similar to that of the wild-type

strain. The filament-inducing properties of Als1p are specific to cells grown on solid media,

because all of the strains described above filamented comparably in liquid media. The data

demonstrates that Als1p promotes filamentation and implicates *ALS1* expression in the

20   regulation of filamentation control pathways. Northern blot analysis of *ALS1* expression in

mutants with defects in each of these pathways, including *efg1/efg1, cph1/cph1, efg1/efg*

cph1/cph1, tup1/tup1, and *cla4/cla4* mutants were performed. Referring to Figure 4A, mutants

in which both alleles of *EFG1* had been disrupted failed to express *ALS1*. Introduction of a copy

of wild-type *EFG1* into the *efg1/efg1* mutant restored *ALS1* expression, though at a reduced

level. See Figure 4B. Also, as seen in Figure 4A, none of the other filamentation regulatory

mutations significantly altered *ALS1* expression (Fig. 4A). Thus, Efg1p is required for *ALS1*

expression.

5   If Efg1p stimulates the expression of *ALS1,* which in turn induces filamentation, the

expression of *ALS1* in the *efg1/efg1* strain should restore filamentation. A functional allele of

ALS1 under the control of the *ADH1* promoter was integrated into the *efg1/efg1* strain. To

investigate the possibility that *ALS1* gene product might complement the filamentation defect in

efg1 null mutant, an Ura *efg1* null mutant was transformed with linearized pAU-1. Ura$^+$ clones

10   were selected and integration of the third copy of *ALS1* was confirmed with PCR using the

primers: 5'-CCGTTTATACCATCCAAATC-3' and 5'-CTACATCCTCCAATGATATAAC-3'.

The resulting strain expressed *ALS1* autonomously and regained the ability to filament on Lee's

agar. See Figures 4B and C. Therefore, Efg1p induces filamentation through activation of *ALS1*

expression.

15   Because filamentation is a critical virulence factor in *C. albicans*, delineation of a

pathway that regulates filamentation has important implications for pathogenicity. Prior to

ALS1, no gene encoding a downstream effector of these regulatory pathways had been identified.

Disruption of two other genes encoding cell surface proteins, *HWP1* AND *INT1*, results in

mutants with filamentation defects. Although *HWP1* expression is also regulated by Efg1p, the

20   autonomous expression of *HWP1* in the *efg1/efg1* mutant fails to restore filamentation.

Therefore Hwp1p alone does not function as an effector of filamentation downstream of *EFG1*.

Also, the regulatory elements controlling *INT1* expression are not know. Thus, Als1p is the first

cell-surface protein identified that functions as a downstream effector of filamentation, thereby

suggesting a pivotal role for this protein in the virulence of *C. albicans*.

The contribution of Als1p to *C. albicans* virulence was tested in a model of

hematogenously disseminated candidiasis, A.S. Ibrahim *et al.*, *Infect. Immun.* **63**, 1993 (1995).

5   Referring to Figure 5A, mice infected with the *als1/als1* null mutant survived significantly

longer than mice infected with the *ALS1/ALS1* strain, the *ALS1/als1* mutant or the *ALS1*-

complemented mutant. After 28 hours of infection, the kidneys of mice infected with the

als1/als1 mutant contained significantly fewer organisms (5.70 ± 0.46 $\log_{10}$ CFU/g) ($P<0.0006$

for both comparisons). No difference was detected in colony counts of organisms recovered

10   from spleen, lungs, or liver of mice infected with either of the strains at any of the tested time

points. These results indicate that Als1p is important for *C. albicans* growth and persistence in

the kidney during the first 28 hours of infection. Referring to Figure 5B, examination of the

kidneys of mice after 28 hours of infection revealed that the *als1/als1* mutant produced

significantly shorter filaments and elicited a weaker inflammatory response than did either the

15   wild-type of *ALS1*-complemented strains. However, by 40 hours of infection, the length of the

filaments and the number of leukocytes surrounding them were similar for all three strains.

The filamentation defect of the *als1/als1* mutant seen on histopathology paralleled the <u>in</u>

<u>vitro</u> filamentation assays on solid media. This mutant showed defective filamentation at early

time points both <u>in vivo</u> and <u>in vitro</u>. This defect eventually resolved with prolonged

20   infection/incubation. These results suggest that a filamentation regulatory pathway that is

independent of *ALS1* may become operative at later time points. The activation of this

alternative filamentation pathway by 40 hours of infection is likely the reason why mice infected

with the *als1/als1* mutant subsequently succumbed in the ensuing 2-3 days.

Collectively, these data demonstrate that *C. albicans ALS1* encodes a cell surface protein that mediates both adherence to endothelial cells and filamentation. Als1p is the only identified downstream effector of any known filamentation regulatory pathway in *C. albicans*. Additionally, Als1p contributes to virulence in hematogenous candidal infection. The cell

5   surface location and dual functionality of Als1p make it an attractive target for both drug and immune-based therapies.

EXAMPLE 3 -- Purification of ALS1 Adhesin Protein

The ALS1 protein synthesized by *E. coli* is adequate as an immunogen. However,

10   eukaryotic proteins synthesized by *E. coli* may not be functional due to improper folding or lack of glycosylation. Therefore, to determine if the ALS1 protein can block the adherence of *C. albicans* to endothelial cells, the protein is, preferably, purified from genetically engineered *C. albicans*.

PCR was used to amplify a fragment of *ALS1*, from nucleotides 52 to 1296. This 1246

15   bp fragment encompassed the N-terminus of the predicted ALS1 protein from the end of the signal peptide to the beginning of the tandem repeats. This region of *ALS1* was amplified because it likely encodes the binding site of the adhesin, based on its homology to the binding region of the *S. cerevisiae Agα1* gene product. In addition, this portion of the predicted ALS1 protein has few glycosylation sites and its size is appropriate for efficient expression in *E. coli*.

20   The fragment of *ALS1* was ligated into pQE32 to produce pINS5. In this plasmid, the protein is expressed under control of the *lac* promoter and it has a 6-hits tag fused to its N-terminus so that it can be affinity purified. We transformed *E. coli* with pINS5, grew it under inducing conditions (in the presence of IPTG), and then lysed the cells. The cell lysate was

passed through a Ni^{2+} -agarose column to affinity purify the *ALS1*-6His fusion protein. This

procedure yielded substantial amounts of *ALS1*-6His. The fusion protein was further purified by

SDS-PAGE. The band containing the protein was excised from the gel so that polyclonal rabbit

antiserum can be raised against it. It will be appreciated by one skilled in the art that the surface

5   adhesin protein according to the invention may be prepared and purified by a variety of known

processes without departing from the spirit of the present invention. The sequence of Als1p is

listed in Figure 7.

EXAMPLE 4 -- Raising Polyclonal Antisera against ALS1 Protein

10   To determine whether antibodies against the ALS1 protein block the adherence of

Candida albicans to endothelial and epithelial cells, and the selected host constituent in vitro,

rabbits were inoculated with *S. cerevisiae* transformed with ALS1 protein. The immunization

protocol used was the dose and schedule used by Hasenclever and Mitchell for production of

antisera that identified the antigenic relationship among various species of *Candida*.

15   Hasenclever, H. F. and W. 0. Mitchell. 1960. Antigenic relationships of *Torulopsis glabrata* and

seven species of the genus *Candida*. J. Bacteriol. 79:677-681. Control antisera were also raised

against *S. cerevisiae* transformed with the empty plasmid. All yeast cells were be grown in

galactose to induce expression of the ALS genes. Before being tested in the adherence

experiments, the serum was heat-inactivated at 56 C to remove all complement activity.

20   Sera from immunized rabbits were absorbed with whole cells of *S. cerevisiae*

transformed with empty plasmid to remove antibodies that are reactive with components of the

yeast other than ALS1 protein. The titer of the antisera was determined by immunofluorescence

using *S. cerevisiae* that express the ALS1 gene. FITC-labeled anti-rabbit antibodies were

purchased from commercial sources (Southern Biotechnology, Inc). Affinity-purified secondary

antibodies were essential because many commercially available sera contain antibodies reactive

with yeast glucan and mannan. The secondary antibodies were pretested using *Candida albicans*

as well as *S. cerevisiae* transformed with the plasmid and were absorbed as needed to remove

5   any anti-*S. cerevisiae* or anti-*Candida* antibodies. Negative controls were 1) preimmune serum,

2) *S. cerevisiae* transformed with the empty plasmid, and 3) *S. cerevisiae* transformed with the

ALS gene but grown under conditions that suppress expression of the ALS gene (glucose).

In addition to the above experiments, Western blotting was used to provide further

confirmation that an antiserum binds specifically to the ALS protein against which it was raised.

10   *S. cerevisiae* transformed with the ALS1 were grown under inducing conditions and their

plasma membranes were isolated by standard methods. Panaretou, B. and P. Piper. 1996.

Isolation of yeast plasma membranes. p. 117-121. In I.H. Evans. (ed.), Yeast Protocols.

Methods in Cell and Molecular Biology. Humana Press, Totowa, New Jersey. Plasma

membranes were also prepared from S, cerevisiae transformed with the empty plasmid and

15   grown under identical conditions. The membrane proteins were separated by SDS-PAGE and

then transferred to PVDF membrane by electroblotting. Harlow, E. and D. Lane. 1988.

Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press. After being blocked

with nonfat milk, the blot was incubated with the ALS antiserum. The preabsorbed antiserum

did not react with proteins extracted from *S. cerevisiae* containing empty plasmid. This

20   antiserum blocked the adherence of S. *cerevisiae* pYF5 (a clone that expresses *Candida albicans*

ALS1) to endothelial cells.

EXAMPLE 5 -- Polyclonal Antibodies Against Specific ALS Proteins Prophylactically

Protect Mice from Mucosal and Hematogenously Disseminated Candidal Infections.

Having identified the antisera that block the adherence of a clone of *S. cerevisiae*

transformed with an ALS1 ALS gene under the above conditions, these antisera were

5   demonstrated to protect mice from intravenous challenge with *Candida albicans*.

The antisera against the ALS proteins were first tested in the murine model of

hematogenously disseminated candidiasis. Affinity-purified anti-ALS antibodies are effective in

preventing adhesion of yeast cells to various substrates (see EXAMPLE 3). Affinity-purification

is useful in this system because antibody doses can be accurately determined. Moreover, the

10   unfractionated antisera will undoubtedly contain large amounts of antibody directed toward

antigens on the *S. cerevisiae* carrier cells. Many of these anti-*Saccharomyces* antibodies would

likely bind to *C. albicans* and make interpretation of the results impossible. Additionally, it is

quite possible that the procedure used to elute antibodies from *S. cerevisiae* that express the

ALS protein may also elute small amounts of yeast mannan or glucan that could have adjuvant-

15   like activity. The immunoaffinity-purified antibodies are further purified before use. They may

also be preabsorbed with mouse splenocytes.

Antibody doses may be administered to cover the range that brackets the levels of serum

antibody that can be expected in most active immunization protocols and to cover the range of

antibody doses that are typically used for passive immunization in murine models of candidiasis.

20   See Dromer, F., J. Charreire, A. Contrepois, C. Carbon, and P. Yeni. 1987, Protection, of mice

against experimental cryptococcosis by *anti-Cryptococcus neofornwns* monoclonal antibody,

Infect. Inimun. 55:749-752; Han, Y. and J. E. Cutler. 1995, Antibody response that protects

against disseminated candidiasis, Infect. Immun. 63:2714-2719; Mukherjee, J., M. D. Scharff,

and A. Casadevall. 1992, <u>Protective murine monoclonal antibodies to *Cryptococcus neofornwns*</u>, Infect. Immun. 60:4534-4541; Sanford, J. E., D. M. Lupan, A. M. Schlageter, and T. R. Kozel. 1990, <u>Passive immunization against *Cryptococcus neoformans* with an isotype-switch family of monoclonal antibodies reactive with cryptococcal polysaccharide</u>, Infect. Inunun. 58:1919-1923.

5   BALB/c mice (female, 7 week old, the NCI) were given anti-ALS that had been absorbed with mouse splenic cells by an intraperitoneal (i.p.) injection. Control mice received prebled serum that had been absorbed with mouse spenic cells, intact anti-ALS serum, or DPBS, respectively. For the pre-absorption, 2 ml of anti-ALS or prebled sera were mixed with 100μl of mouse (BALB/c, 7 weeks old female, NCI) splenic cells (app. 9×10^6 cells per ml) at room temperature

10   for 20 minutes. The mixture was washed with warm sterile DPBS by centrifugation (@ 300 xg) for 3 minutes. This procedure was repeated three times. The volume of i.p. injection was 0.4 ml per mouse. Four hours later, the mice were challenged with *C. albicans* (strain CA-1; 5×10^5 hydrophilic yeast cells per mouse) by i.v. injection. Then, their survival times were measured. See Figure 6.

15   Previous studies have shown that antibodies administered via the intraperitoneal route are rapidly (within minutes) and almost completely transferred to the serum (Kozel and Casadevall, unpublished observations). As a control for effects of administering the antibody preparations, a parallel group of mice were treated with antibodies isolated from pre-immune serum that has been absorbed with *S. cerevisiae* transformed with the ALS gene. The survival time and

20   numbers of yeast per gram of kidney were measured. Again, referring to Figure 6, mice infected intravenously with 10^6 blastopores of ALS1 null mutant had a longer median survival time when compared to mice infected with *Candida albicans* CAI-12 or *Candida albicans* in which one allele of the ALS1 had been deleted (p=0.003).

These results indicate that immunotherapeutic strategies using the ALS1 proteins as a

vaccine have a protective prophylactic effect against disseminated candidiasis.

The particular examples set forth herein are instructional and should not be interpreted as

limitations on the applications to which those of ordinary skill are able to apply this invention.

5   Modifications and other uses are available to those skilled in the art which are encompassed

within the spirit and scope of the following claims.

CLAIMS

I claim:

1.   A vaccine comprising:

5   a pharmaceutical composition comprising isolated and purified cell surface adhesin

protein obtained from a *Candida* strain.

2.   The vaccine of Claim 1, wherein the cell surface adhesin protein is *Candida*

albicans ALS1 adhesion protein (Als1p).

3.   The vaccine of Claim 2, wherein the protein is a fragment encompassing the N-

10   terminus of Als1p and containing the adhesin binding site.

4.   The vaccine of Claim 1, wherein said cell surface adhesin protein is derived from

a Candidal strain selected from the group consisting of *Candida krusei, Candida tropicalis, and*

Candida, parapsilosis.

5.   A method for treatment and prevention of disseminated candidiasis, comprising:

15   administering a vaccine comprised of a pharmaceutically effective amount of an ALS1

adhesion protein of *Candida albicans* in an amount elicit to an immune response.

6.   The method of Claim 5, wherein said step of administering vaccine comprises the

step of using active immunization through administration of the *Candida albicans* ALS1 gene
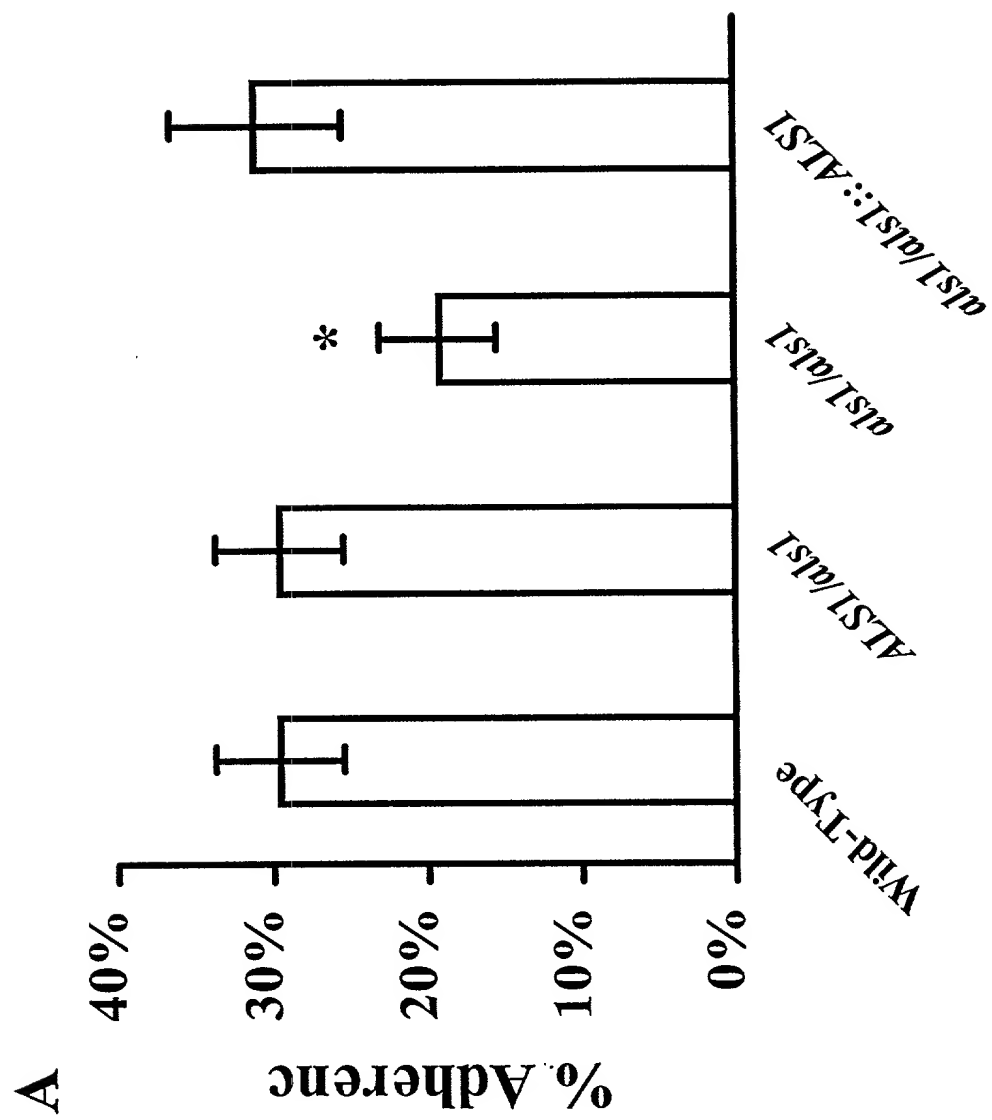
product.

20   7.   The method of Claim 5, wherein said step of administering vaccine comprises

passive immunization through administration of antiserum to ASL1 gene product.

8.   The method of Claim 5, wherein said step of administering vaccine further

comprises the step of using a combination of active immunotherapy with the ALS1 gene product

and passive immunotherapy with antiserum to the ALS1 gene product.

ABSTRACT:

A *Candida albicans* bloodstream infections cause significant morbidity and mortality in hospitalized patients. Filament formation and adherence to host cells are critical virulence factors of *C. albicans*. Multiple filamentation regulatory pathways have been discovered,

5   however the downstream effectors of these regulatory pathways remain unknown. The cell surface protein, Als1p, is a downstream effector of the filamentation regulatory pathway and is regulated by Efg1p. Als1p mediates adherence to endothelial cells in vitro and is required for virulence. The blocking of adherence by the organism is described resulting from the use of a composition and method disclosed herein. Specifically, a pharmaceutical composition

10   comprised of a gene product from the ALS1 gene family is administered as a vaccine to generate an immune response capable of blocking adherence of the organism.
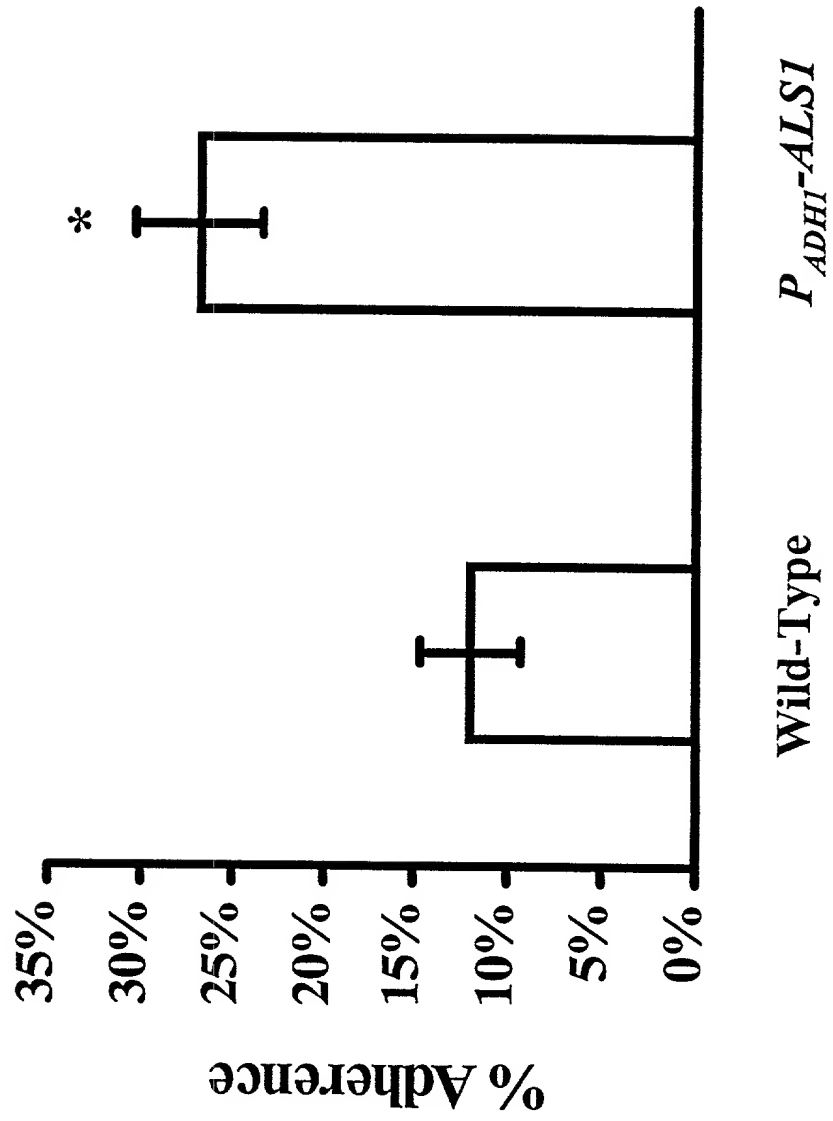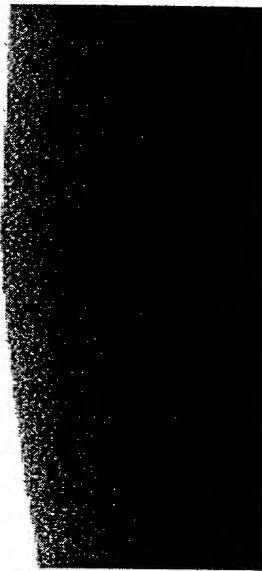
FIGURE 1A

FIGURE 1B

FIGURES 2A, 2B, 2C, 2D

FIGURE 3A

A

Wild-Type   als1/als1   als1/als1::ALS1

P_{ADH1}-ALS1

Wild-Type

B

FIGURES 4A

B

efg1/efg1

*efg1/efg1, P*_{*ADH1*} *ALS1*
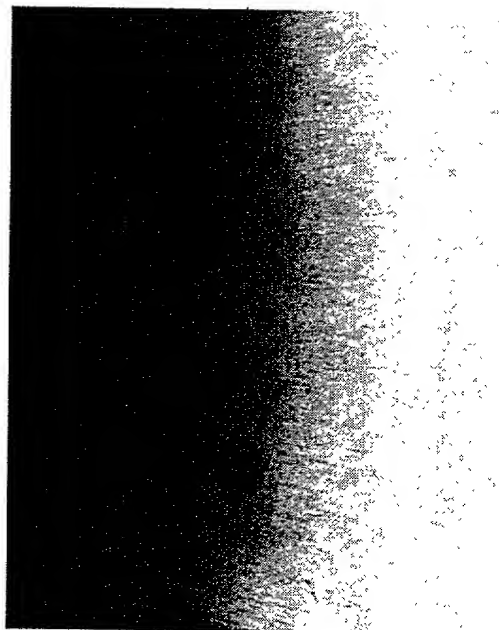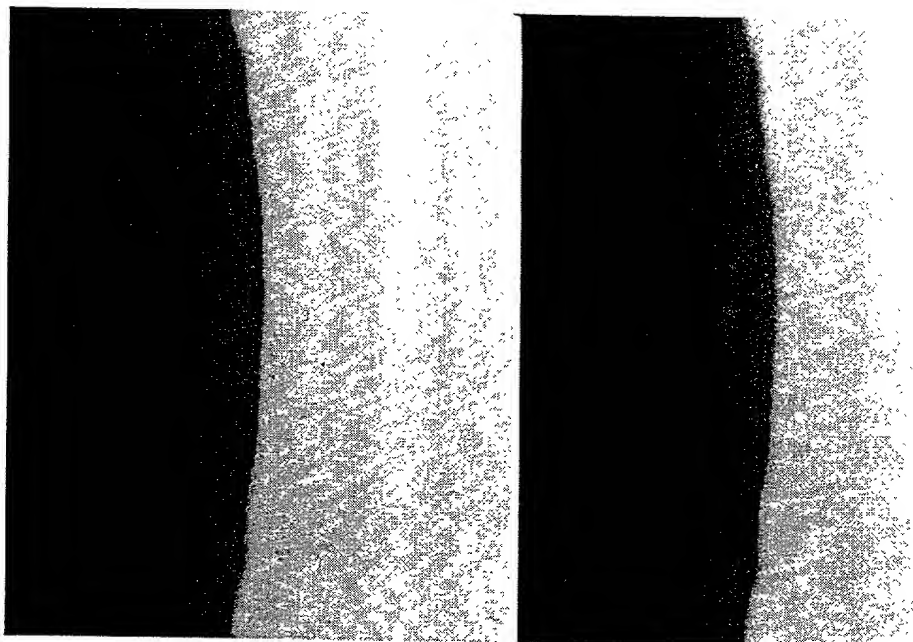
A

% Survival

Days

Wild-Type
ALS1/als1
als1/als1
als1/als1::ALS1

*

B

a   Wild-Type   als1/als1   als1/als1::ALS1

b   Wild-Type   als1/als1   als1/als1::ALS1

Prophylactic effect of antiALS antibody against disseminated candidiasis

	MST (days)
—○— DPBS	11.4 ± 3.3
—□— NRS (PB)[1]	15.4 ± 4.4
—△— S[2]-antiALS	22.0 ± 4.5
—▽— I[3]-antiALS	16.8 ± 2.6

FIGURE 6

```
1     ATGCTTCAACAATTTACATTGTTATTCCTATATTTGTCAATTGCAAGTGCAAAGACAATC
1      M  L  Q  Q  F  T  L  L  F  L  Y  L  S  I  A  S  A  K  T  I

61    ACTGGTGTTTTTGATAGTTTTAATTCATTAACTTGGTCCAATGCTGCTAATTATGCTTTC
21     T  G  V  F  D  S  F  N  S  L  T  W  S  N  A  A  N  Y  A  F

121   AAAGGGCCAGGATACCCAACTTGGAATGCTGTTTTGGGTTGGTCCTTAGATGGTACCAGT
41     K  G  P  G  Y  P  T  W  N  A  V  L  G  W  S  L  D  G  T  S

181   GCCAATCCAGGGGATACATTCACATTGAATATGCCATGTGTGTTTAAATATACTACTTCA
61     A  N  P  G  D  T  F  T  L  N  M  P  C  V  F  K  Y  T  T  S

241   CAAACATCTGTTGATTTAACTGCCGATGGTGTTAAATATGCTACTTGTCAATTTTATTCT
81     Q  T  S  V  D  L  T  A  D  G  V  K  Y  A  T  C  Q  F  Y  S

301   GGTGAAGAATTCACAACTTTTTCTACATTAACATGTACTGTGAACGACGCTTTGAAATCA
101    G  E  E  F  T  T  F  S  T  L  T  C  T  V  N  D  A  L  K  S

361   TCCATTAAGGCATTTGGTACAGTTACTTTACCAATTGCATTCAATGTTGGTGGAACAGGT
121    S  I  K  A  F  G  T  V  T  L  P  I  A  F  N  V  G  G  T  G

421   TCATCAACTGATTTGGAAGATTCTAAATGTTTTACTGCTGGTACCAATACAGTCACATTT
141    S  S  T  D  L  E  D  S  K  C  F  T  A  G  T  N  T  V  T  F

481   AATGATGGTGATAAAGATATCTCAATTGATGTTGAGTTTGAAAAGTCAACCGTTGATCCA
161    N  D  G  D  K  D  I  S  I  D  V  E  F  E  K  S  T  V  D  P

541   AGTGCATATTTGTATGCTTCCAGAGTTATGCCAAGTCTCAATAAGGTCACAACTCTTTTT
181    S  A  Y  L  Y  A  S  R  V  M  P  S  L  N  K  V  T  T  L  F

601   GTGGCACCACAATGTGAAAATGGTTACACATCTGGTACAATGGGGTTCTCCAGTAGTAAC
201    V  A  P  Q  C  E  N  G  Y  T  S  G  T  M  G  F  S  S  N

661   GGTGACGTTGCTATTGATTGCTCAAATATTCATATTGGTATCACAAAAGGATTAAATGAT
221    G  D  V  A  I  D  C  S  N  I  H  I  G  I  T  K  G  L  N  D

721   TGGAATTATCCGGTTTCATCTGAATCATTTAGTTACACTAAAACTTGTACATCTAATGGA
241    W  N  Y  P  V  S  S  E  S  F  S  Y  T  K  T  C  T  S  N  G

781   ATTCAGATTAAATATCAAAATGTACCTGCTGGTTATCGTCCATTTATTGATGCTTATATT
261    I  Q  I  K  Y  Q  N  V  P  A  G  Y  R  P  F  I  D  A  Y  I

841   TCTGCTACAGATGTTAACCAATATACTTTAGCATATACCAATGATTATACTTGTGCTGGC
281    S  A  T  D  V  N  Q  Y  T  L  A  Y  T  N  D  Y  T  C  A  G

901   AGTCGTCTGCAAAGTAAACCTTTCACTTTAAGATGGACTGGATACAAGAATAGTGATGCC
301    S  R  L  Q  S  K  P  F  T  L  R  W  T  G  Y  K  N  S  D  A

961   GGATCTAACGGTATTGTCATTGTTGCTACAACTAGAACAGTTACAGACAGTACCACTGCT
321    G  S  N  G  I  V  I  V  A  T  T  R  T  V  T  D  S  T  T  A

1021  GTCACTACTTTACCATTCAATCCAAGTGTTGATAAAACCAAAACAATCGAAATTTTGCAA
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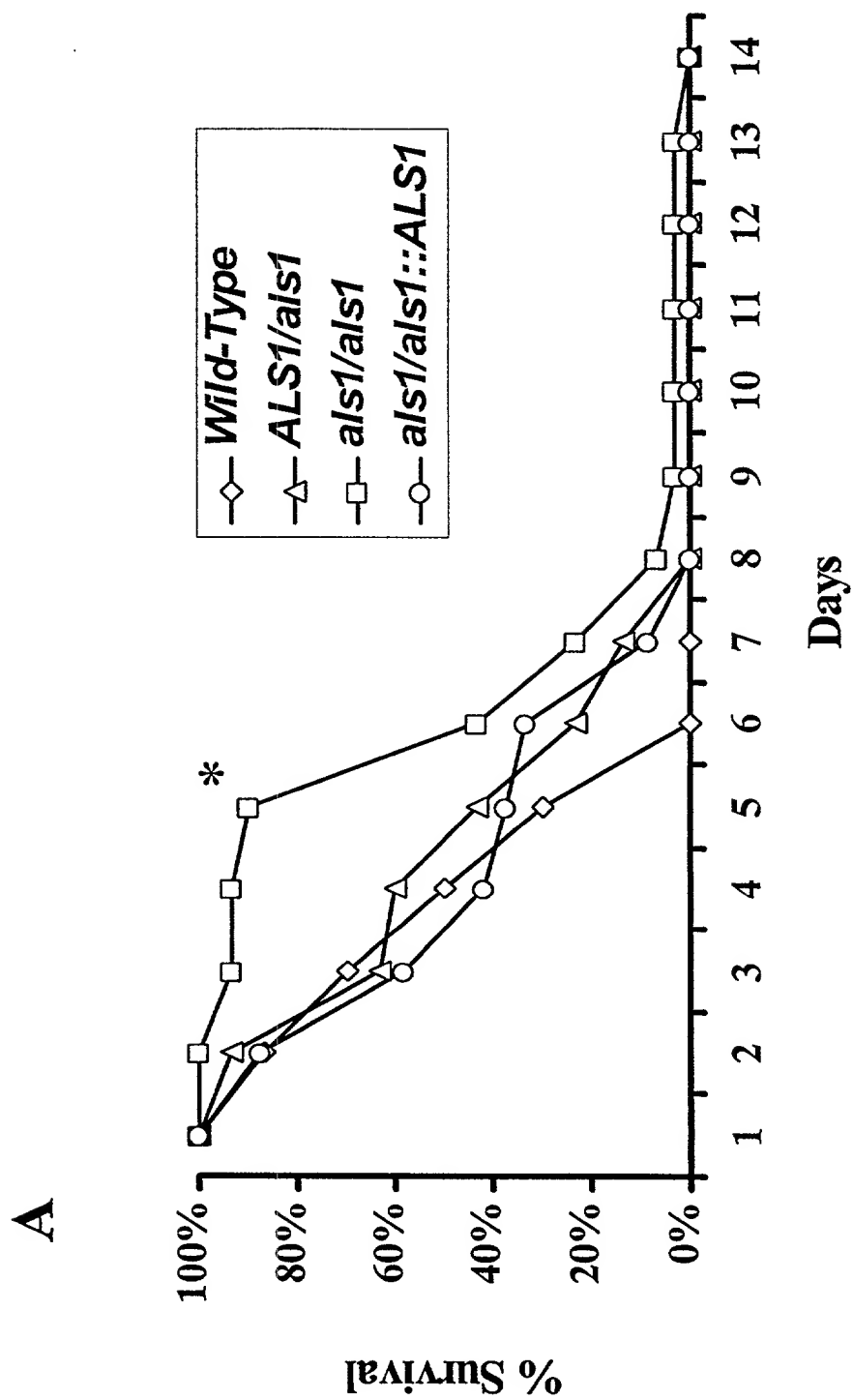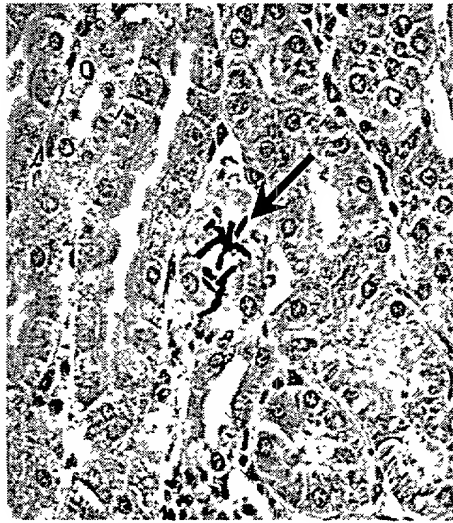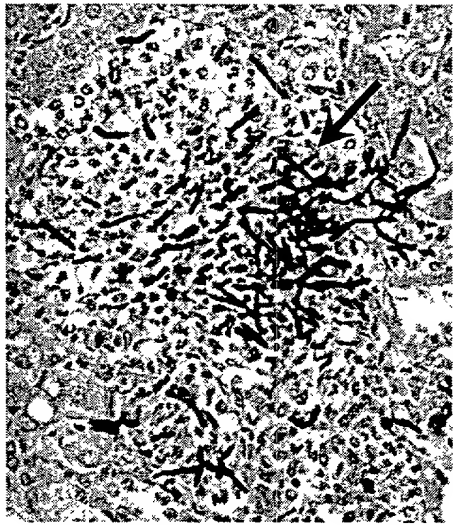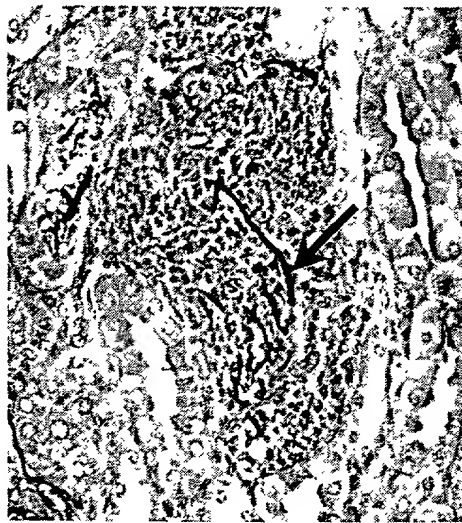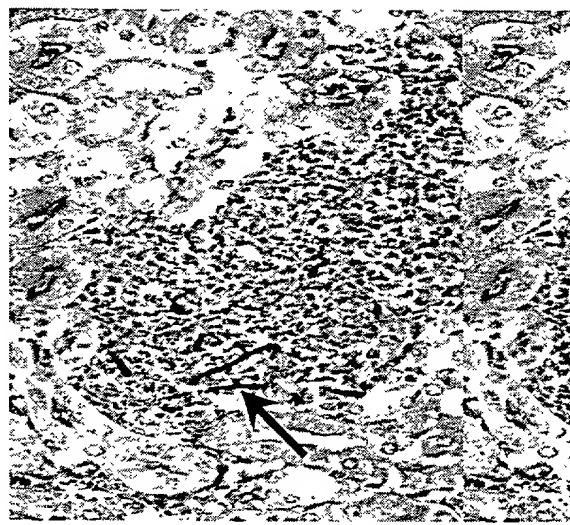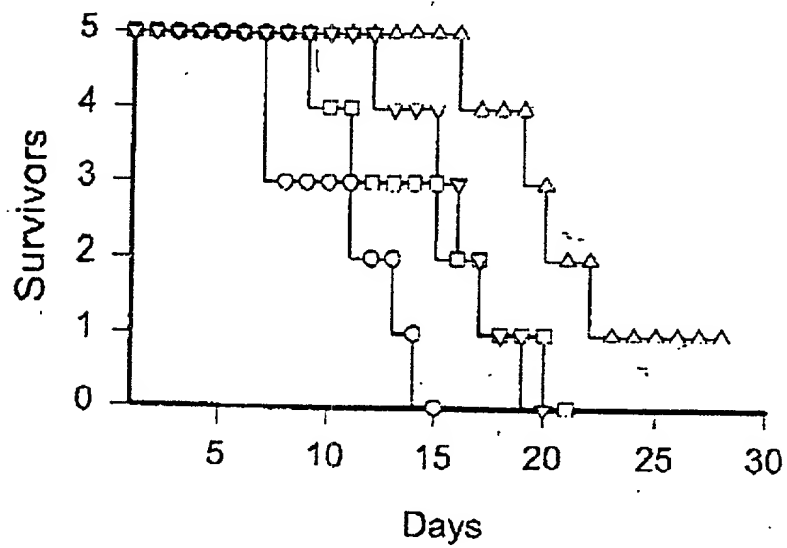
FIGURE 7

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341           V   T   T   L   P   F   N   P   S   V   D   K   T   K   T   I   E   I   L   Q

1081      CCTATTCCAACCACTACCATCACAACTTCATATGTTGGTGTGACTACTTCCTATCTGACT
361          P   I   P   T   T   T   I   T   T   S   Y   V   G   V   T   T   S   Y   L   T

1141      AAGACTGCACCAATTGGTGAAACAGCTACTGTTATTGTTGATGTGCCATATCATACTACC
381          K   T   A   P   I   G   E   T   A   T   V   I   V   D   V   P   Y   H   T   T

1201      ACAACTGTTACCAGTGAATGGACAGGAACAATCACTACCACCACAACTCGTACCAATCCA
401          T   T   V   T   S   E   W   T   G   T   I   T   T   T   T   T   R   T   N   P

1261      ACTGATTCAATTGACACAGTGGTGGTACAAGTTCCACTGCCAAATCCAACTGTTAGTACT
421          T   D   S   I   D   T   V   V   V   Q   V   P   L   P   N   P   T   V   S   T

1321      ACTGAATATTGGTCTCAGTCCTTTGCTACAACCACTACAGTTACTGCTCCTCCAGGTGGT
441          T   E   Y   W   S   Q   S   F   A   T   T   T   T   V   T   A   P   P   G   G

1381      ACCGATACTGTGATTATCAGAGAGCCACCAAACCATACTGTCACTACTACTGAATATTGG
461          T   D   T   V   I   I   R   E   P   P   N   H   T   V   T   T   T   E   Y   W

1441      TCACAATCCTTTGCTACTACTACTACTGTTACTGCTCCTCCAGGTGGTACTGACTCAGTA
481          S   Q   S   F   A   T   T   T   T   V   T   A   P   P   G   G   T   D   S   V

1501      ATTATCAGAGAACCACCAAATCCAACTGTCACTACAACCGAGTATTGGTCTCAATCCTTT
501          I   I   R   E   P   P   N   P   T   V   T   T   T   E   Y   W   S   Q   S   F

1561      GCTACTACTACTACAGTTACTGCTCCTCCAGGTGGTACTGACTCAGTAATTATCAGAGAA
521          A   T   T   T   T   V   T   A   P   P   G   G   T   D   S   V   I   I   R   E

1621      CCTCCAAACCCAACTGTCACCACCACTGAATATTGGTCCCAATCTTACGCAACCACAACT
541          P   P   N   P   T   V   T   T   T   E   Y   W   S   Q   S   Y   A   T   T   T

1681      ACTGTGACTGCTCCTCCAGGAGGCACTGACTCAGTAATTATCAGAGAACCACCAAACCAC
561          T   V   T   A   P   P   G   G   T   D   S   V   I   I   R   E   P   P   N   H

1741      ACTGTCACTACTACTGAATACTGGTCACAATCATATGCCACCACTACCACTGTAACTGCA
581          T   V   T   T   T   E   Y   W   S   Q   S   Y   A   T   T   T   T   V   T   A

1801      CCACCAGGTGGTACTGACACTGTTATCATTAGAGAGCCACCAAACCACACTGTCACTACT
601          P   P   G   G   T   D   T   V   I   I   R   E   P   P   N   H   T   V   T   T

1861      ACTGAGTATTGGTCTCAATCGTTTGCTACTACCACAACTGTAACTGGTCCACCAAGTGGC
621          T   E   Y   W   S   Q   S   F   A   T   T   T   T   V   T   G   P   P   S   G

1921      ACTGATACTGTTATCATTAGGGAACCACCAAACCCAACTGTCACCACTACTGAATACTGG
641          T   D   T   V   I   I   R   E   P   P   N   P   T   V   T   T   T   E   Y   W

1981      TCTCAATCATATGCAACCACTACTACCATTACCGCTCCACCTGGTGAAACTGATACCGTT
661          S   Q   S   Y   A   T   T   T   T   I   T   A   P   P   G   E   T   D   T   V

2041      CTTATCAGAGAGCCACCAAACCATACTGTCACTACTACTGAATACTGGTCTCAATCATAT
681          L   I   R   E   P   P   N   H   T   V   T   T   T   E   Y   W   S   Q   S   Y

2101      GCTACAACCACCACTGTTACTGCACCACCTGGTGAAACCGATACCGTTCTTATCAGAGAG
701          A   T   T   T   T   V   T   A   P   P   G   E   T   D   T   V   L   I   R   E

2161      CCACCAAACCATACTGTCACTACTACTGAATACTGGTCTCAATCATATGCTACAACCACC
```

FIGURE 7

```
721        P  P  N  H  T  V  T  T  T  E  Y  W  S  Q  S  Y  A  T  T  T

2221    ACTGTTACTGCACCACCAGGTGGTACCGATACTGTTATCATTAGAGAGCCACCAAATCCA
741        T  V  T  A  P  P  G  G  T  D  T  V  I  I  R  E  P  P  N  P

2281    ACAGTTACTACTACTGAATATTGGTCACAATCATTTGCCACAACCACCACAGTTACTGCT
761        T  V  T  T  T  E  Y  W  S  Q  S  F  A  T  T  T  T  V  T  A

2341    CCTCCAGGTGGTACTGACACTGTGATTATCTATGAAAGCATGTCAAGTTCAAAGATTTCT
781        P  P  G  G  T  D  T  V  I  I  Y  E  S  M  S  S  S  K  I  S

2401    ACATCCTCCAATGATATAACCAGTATCATTCCATCATTTTCCCGTCCTCATTATGTCAAC
801        T  S  S  N  D  I  T  S  I  I  P  S  F  S  R  P  H  Y  V  N

2461    AGCACAACCTCCGATTTGTCAACATTTGAATCTTCATCCATGAATACTCCTACTTCTATC
821        S  T  T  S  D  L  S  T  F  E  S  S  S  M  N  T  P  T  S  I

2521    AGTAGTGATGGTATGTTGTTGTCTTCTACAACTTTGGTTACTGAATCAGAAACAACTACA
841        S  S  D  G  M  L  L  S  S  T  T  L  V  T  E  S  E  T  T  T

2581    GAACTGATTTGCAGTGATGGTAAAGAGTGTTCTAGATTGTCCAGTTCTTCTGGTATTGTC
861        E  L  I  C  S  D  G  K  E  C  S  R  L  S  S  S  S  G  I  V

2641    ACAAATCCAGATAGCAATGAATCCTCAATCGTAACTAGTACTGTTCCTACTGCAAGTACA
881        T  N  P  D  S  N  E  S  S  I  V  T  S  T  V  P  T  A  S  T

2701    ATGTCTGATTCACTTTCTTCAACTGATGGTATTAGTGCTACATCTTCTGATAATGTTTCA
901        M  S  D  S  L  S  S  T  D  G  I  S  A  T  S  S  D  N  V  S

2761    AAATCAGGAGTATCAGTTACAACCGAAACTTCTGTTACAACTATTCAAACTACTCCAAAC
921        K  S  G  V  S  V  T  T  E  T  S  V  T  T  I  Q  T  T  P  N

2821    CCATTATCATCTTCAGTGACATCATTGACTCAGTTGTCTTCAATTCCAAGTGTTTCAGAA
941        P  L  S  S  S  V  T  S  L  T  Q  L  S  S  I  P  S  V  S  E

2881    AGTGAAAGTAAAGTTACATTTACAAGCAATGGAGACAACCAAAGTGGTACTCATGATTCA
961        S  E  S  K  V  T  F  T  S  N  G  D  N  Q  S  G  T  H  D  S

2941    CAATCTACTTCCACTGAAATTGAAATTGTAACAACCAGTTCTACTAAAGTTTTACCACCT
981        Q  S  T  S  T  E  I  E  I  V  T  T  S  S  T  K  V  L  P  P

3001    GTCGTTTCTTCTAATACTGATTTGACTAGTGAACCAACAAATACCAGAGAACAACCAACT
1001       V  V  S  N  T  D  L  T  S  E  P  T  N  T  R  E  Q  P  T

3061    ACATTATCAACTACTTCAAACTCCATCACTGAAGATATCACCACATCTCAACCTACAGGT
1021       T  L  S  T  T  S  N  S  I  T  E  D  I  T  T  S  Q  P  T  G

3121    GATAATGGAGACAATACTTCATCAACCAATCCAGTTCCAACTGTGGCAACAAGTACTTTA
1041       D  N  G  D  N  T  S  S  T  N  P  V  P  T  V  A  T  S  T  L

3181    GCATCTGCAAGTGAAGAAGACAACAAAAGCGGTTCTCATGAATCAGCATCCACAAGTTTG
1061       A  S  A  S  E  E  D  N  K  S  G  S  H  E  S  A  S  T  S  L

3241    AAACCAAGTATGGGTGAAAATTCTGGATTAACTACTTCTACTGAAATTGAAGCTACAACA
1081       K  P  S  M  G  E  N  S  G  L  T  T  S  T  E  I  E  A  T  T

3301    ACCAGTCCTACAGAAGCTCCATCACCTGCTGTTTCTTCTGGTACTGATGTAACTACTGAA
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FIGURE 7

```
1101        T   S   P   T   E   A   P   S   P   A   V   S   S   G   T   D   V   T   T   E

3361     CCAACTGATACTAGAGAACAACCTACTACATTATCAACTACTTCAAAAACAAACAGTGAA
1121       P   T   D   T   R   E   Q   P   T   T   L   S   T   T   S   K   T   N   S   E

3421     CTGGTTGCTACTACACAAGCTACTAATGAAAATGGTGGTAAATCTCCATCAACTGATTTA
1141       L   V   A   T   T   Q   A   T   N   E   N   G   G   K   S   P   S   T   D   L

3481     ACATCAAGCTTGACAACAGGCACCTCAGCATCTACAAGTGCTAATAGCGAACTTGTTACT
1161       T   S   S   L   T   T   G   T   S   A   S   T   S   A   N   S   E   L   V   T

3541     AGTGGATCTGTTACTGGTGGAGCTGTTGCCAGTGCTTCAAATGATCAATCACATTCTACT
1181       S   G   S   V   T   G   G   A   V   A   S   A   S   N   D   Q   S   H   S   T

3601     TCTGTTACCAACAGCAACAGCATTGTATCTAATACCCCACAAACTACATTGAGTCAACAA
1201       S   V   T   N   S   N   S   I   V   S   N   T   P   Q   T   T   L   S   Q   Q

3661     GTTACCTCATCCTCACCTTCAACCAACACATTCATTGCTTCTACATACGATGGCTCTGGT
1221       V   T   S   S   P   S   T   N   T   F   I   A   S   T   Y   D   G   S   G

3721     TCTATTATCCAACATTCTACTTGGTTGTACGGTTTGATCACATTATTGTCCTTGTTCATT
1241       S   I   I   Q   H   S   T   W   L   Y   G   L   I   T   L   L   S   L   F   I

3781     TAGTGA
1261       *   *
```

FIGURE 7